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In vitro Evidence for a Neural Factor(s) Involved in the Proliferation of Adenohypophysial Primordial Cells in Fetal Rats

Yuichi G. Watanabe and Manabu Shirai

Department of Biology, Faculty of Science, Niigata University
Niigata 950-21, Japan

ABSTRACT—Our previous *in vitro* data have shown that proliferative activity of adenohypophysial primordial cells of the rat drops markedly after removal of the developing diencephalic floor. In this study, we transplanted the diencephalic floor to an area of the adenohypophysial primordium that had originally not been in contact with the brain. This transplantation experiment was carried out on day 13.5 of fetal age, when most of the proliferating adenohypophysial cells are directed toward the developing brain. DNA-replicating cells in the culture explants were revealed one day later by incorporation of bromodeoxyuridine (BrDU) followed by its detection by the use of a monoclonal antibody.

The results of the transplantation experiments differed, depending on which part of the primordium came in contact with the neural tissue. If the diencephalic floor was attached to the dorsal half of the adenohypophysial primordium, a new brain-dependent pattern of cell proliferation took place in the explants. Transplantation of the diencephalic floor, on the other hand, to the ventral half of the developing adenohypophysis resulted in a decrease in the proliferative rate of the adenohypophysial cells. The cell proliferative activity of brain-deprived adenohypophysial explants was very low. Thus the results of our study show that the presumptive neural factor(s) contained in the developing brain affects only dorsally located cells of the adenohypophysial primordium.

INTRODUCTION

The epithelio-mesenchymal interaction is the most well known and extensively studied subject that is essential for morphogenesis of most organs of epithelial origin. Whereas development of some epithelial tissues is under the inductive influence of the brain. For example, morphogenesis of the lens depends on the presence of the optic vesicle, or an outpocketing of the diencephalic wall [1, 9, 23]. Since the adenohypophysis arises in close association with the diencephalic floor [14], the possibility of a neural influence was investigated; and the results revealed a crucial role of the diencephalic floor in both proliferation [15] and differentiation [20, 21] of adenohypophysial primordial cells in the rat. At the early stage of development, cell proliferation occurs predominantly in the dorsal half of the adenohypophysial primordium [15]. This may be interpreted as 1) the restricted diffusion of the presumptive neural factor or 2) the different responsiveness of primordial cells to the neural factor. To decide which of the two holds true, we investigated whether the transplantation of the diencephalic floor changes the pattern of cell proliferation in the developing adenohypophysis.

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MATERIALS AND METHODS

Animals

Sexually mature rats of the Sprague-Dawley strain were mated at night. If spermatozoa were found in the vaginal smears the next morning, noon of that day was designated as day 0.5 of gestation.

Organ culture

Pregnant rats on day 13.5 were anesthetized

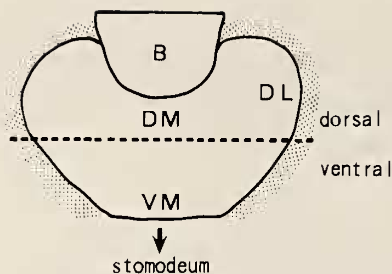


Fig. 1. Diagram illustrating the topological relationship between the adenohypophysial primordium and brain (B) or mesenchyme (stippled area), and the sites (DL, VM) to which brain or mesenchymal tissue was transplanted. DM, DL and VM indicate the dorso-medial, dorso-lateral and ventro-medial regions of the primordium, respectively.

with ketamine hydrochloride solution. After laparotomy, fetuses were removed one by one and the basal part of the diencephalic vesicle with the adenohypophysial primordium (Fig. 1) was isolated in Ca- and Mg-free Hanks solutions with the aid of a dissecting microscope. Primordial tissue was then treated with a mixture of an equal part of 0.3% collagenase (Sigma, type V) and serum-free MEM (Gibco, Grand Island, New York) at room temperature. Separation of brain and/or mesenchyme was done under a dissecting microscope with the aid of fine watchmaker's forceps. After about 10 minutes of enzyme treatment, different combinations of culture explants were prepared as shown in Table 1. On occasion, a fragment of brain tissue was left to know the original brain-adenohypophysial contacting site. Each explant was placed on a piece of cellulose acetate membrane and cultured in a dish for organ culture (Falcon, no. 3037). As described elsewhere [15], a mark was made on the acetate membrane so that the orientation of the culture explants in histological sections would be known. Cultures were maintained in αMEM containing 0.1% fetal calf serum and 30 mM glucose. One day after organ culture, bromodeoxyuridine (BrDU) was added to the medium at a concentration of 6 μg/ml. Three hours later explants were fixed overnight in Bouin's solution. The cellulose acetate membranes were removed in the course of dehydration in ethanol.

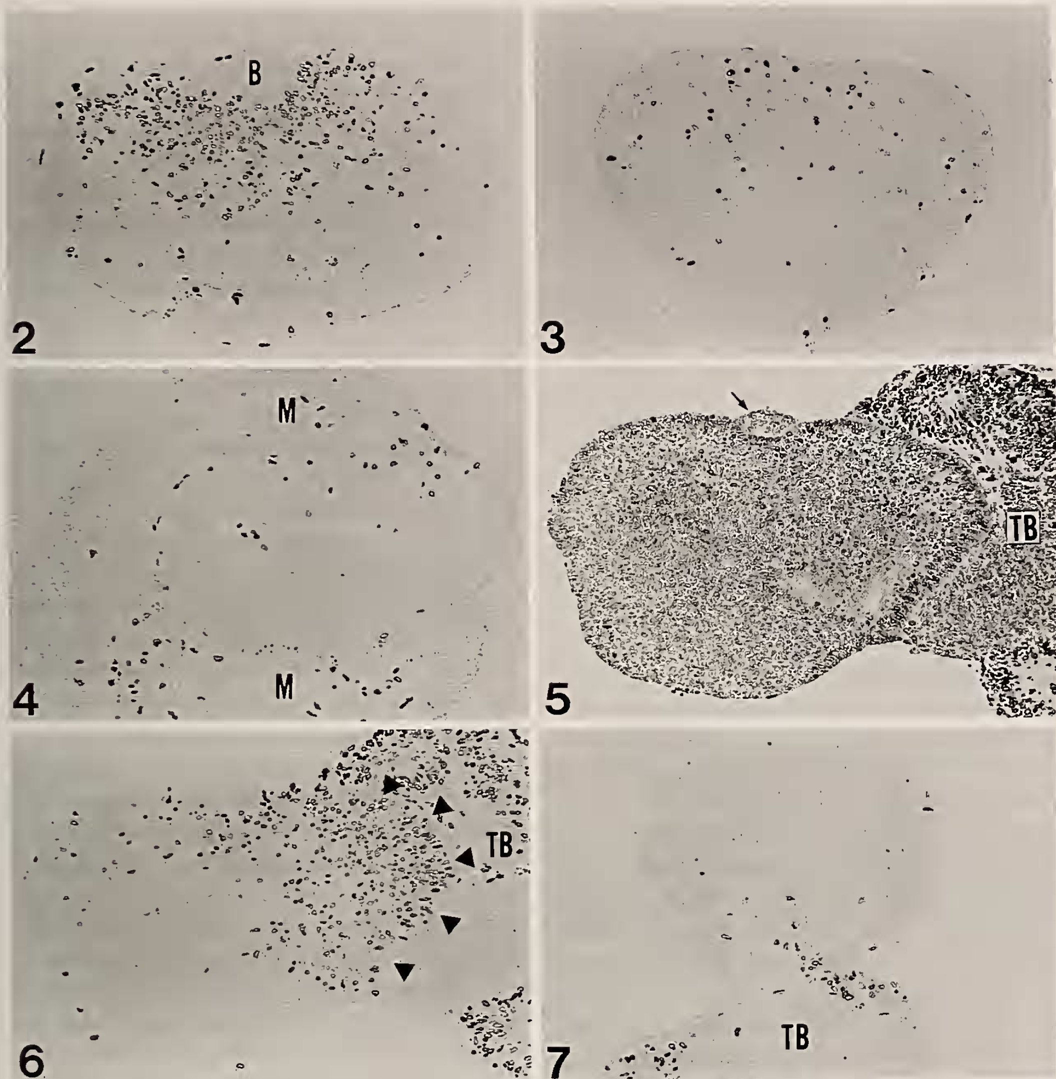
Immunohistochemistry

After embedding in Paraplast, sections were cut at 2 μm with the use of glass knives. Deparaffinized sections were incubated with a monoclonal antibody against BrDU for 1 hr and then with peroxidase-conjugated anti-mouse IgG for 30 min. The reaction product was then visualized with 3,3′-diaminobenzidine tetrahydrochloride solution containing H_2O_2. The antisera and BrDU solution were purchased from Amersham(UK). Some sections were simply stained in Caracci's hematoxylin solution to confirm the results of the transplantation. In each explant, the largest profile of section was selected and the number of BrDU-labelled nuclei was counted at a magnification of 400×.

Table 1. Incidence of BrDU-labelled cells in adenohypophysial explants in different culture conditions

Tissues co-cultured		n	Dorsal half		Ventral half
Brain	Mesenchyme		Left	Right	
intact	—	6	133.0±7.6	111.3±10.4	18.9±2.5
—	—	4	15.5±1.3	12.0± 2.3	5.0±2.4
transplanted to area DL	—	3	35.3±4.3	266.0±20.5*	10.3±3.4
transplanted to area VM	—	3	8.3±3.2	6.3± 0.9	8.7±1.2*
—	intact (area DL-VM)	3	12.0±4.4	7.3± 1.5	4.3±1.9

Values are means±S.E.
*, brain-transplanted portion.
For explanations of areas DL and VM, see Figure 1.



FIGS. 2–7. Sections through rat adenohypophysial primordia separated on fetal day 13.5 and cultured for 2 days. All of these sections except that in Figure 5 were immunostained with anti-BrDU that had been incorporated 3 h before fixation. Figures are all oriented with the dorsal side of the primordium at the top. ×100. FIG. 2. Explant cultured with a part of brain (B). Most of BrDU-labelled cells are seen in the dorsal half of the primordium. FIG. 3. Explant cultured without brain tissue. The incidence of BrDU-labelled cells is low. FIG. 4. Explant cultured with mesenchyme (M). Adenohypophysis contains a small number of labelled cells. FIG. 5. Explant after transplantation of the brain (TB) to the dorso-lateral region of the primordium. In this explant a small fragment of brain tissue (arrow) was intentionally left to confirm the original site where the brain was attached to adenohypophysis. Hematoxylin stain. FIG. 6. A consecutive section of the explant shown in Figure 5. BrDU-labelled cells are observed toward the transplanted brain (TB). The boundary of the brain and adenohypophysis is shown by arrowheads. FIG. 7. Explant after transplantation of the brain (TB) to the ventro-medial part of the primordium. The incidence of labelled cells is very low.

The incidence of labelled cells was compared in different three regions, or the ventral and dorsal (right and left) halves of adenohypophysial tissue.

RESULTS

When the diencephalic floor was left intact, many BrDU-labelled cells were observed in adenohypophysial tissue (Table 1). Most of these labelled cells were in the dorso-medial (DM) region of the adenohypophysis, where the brain was attached (Fig. 2). In hematoxylin-stained sections, mitotic figures were frequently seen. Enzymatic removal of the diencephalic floor and mesenchymal tissue resulted in a marked decrease in the rate of cell labelling (Fig. 3 and Table 1). This profound decrease in the incidence of labelled cells was also observed in explants with mesenchyme left intact (Fig. 4 and Table 1). In these explants, mesenchymal tissue was in contact with most of the margin of the adenohypophysis including the dorso-medial region where the brain was attached. Although labelled cells were slightly more numerous in number in the dorsal part of the adenohypophysis than elsewhere, the overall labelling incidence was far less when compared with those explants maintained with the brain.

Transplantation of the diencephalic floor to the dorso-lateral (DL) part of the adenohypophysial primordium (Fig. 5) caused a remarkable change in the pattern of cell proliferation. The dorso-medial part (DM) of the adenohypophysial tissue, where the brain was originally attached, contained only a small number of labelled cells. In contrast, many labelled cells were seen in the new brain-contact area of adenohypophysial tissue (Fig. 6 and Table 1). This change in localization of proliferating cells was observed only when the diencephalic floor was transplanted to the dorsal half of the adenohypophysial primordium. The incidence of BrDU-labelled cells was quite low when the brain was removed and attached to the ventro-medial area (VM) of the primordium (Fig. 7 and Table 1).

DISCUSSION

There is a close similarity between the development of the adenohypophysis and lens. The rudiments of both of these organs thicken and invaginate keeping in contact with the outgrowth of the diencephalic vesicle. Development of the lens has been studied more frequently than that of the adenohypophysis. The experimental evidence indicates that the optic vesicle of the diencephalon induces formation of the lens in vertebrates [1, 7, 9, 10, 18]. On the other hand, mesenchymal tissue is believed to hinder normal morphogenesis of the lens. In fact mesenchymal cells located between the optic vesicle and lens placode become necrotic and are resorbed in a normal mouse fetus [17]. Further, in anophthalmic mice mesenchymal cells fail to degenerate and consequently hinder the intimate contact of the optic vesicle and lens placode [17] which is necessary for normal development of the lens.

It has been generally accepted that an early event in the differentiation of a rudiment exposed to an inductive stimulus is an increase in the rate of mitosis of the primordial cells [6, 12, 13, 24]. In the present study, the rate of proliferation of adenohypophysial primordial cells increased markedly in the presence of the brain.

In a series of *in vitro* experiments using fetal rats, we have found that the developing diencephalic floor is essential for the proliferation and differentiation of adenohypophysial cells [15, 16, 20, 21]. The results of the present study further suggest that the developing diencephalon contains a neural factor that stimulates the early proliferation of the adenohypophysial primordial cells. Although it is not known how this neural factor reaches the adenohypophysial tissue, it seems of interest to compare the pattern of cell proliferation of our explants with that of growth of other primordia that also require an inductive influence *in vitro*. In a transfilter culture experiment, cell proliferation of metanephrogenic mesenchyme was not confined to the area of the membrane filter whose undersurface faced the inductor; thus, ^{3}H-incorporating metanephrogenic cells were distributed homogeneously in these explants [13]. On the other hand, most of ^{3}H-thymidine labelled cells in the stimulated pancreatic primordium were localized near the peripheral region of the explants, without any topological relation to the

inductive stimulus [24]. In view of these observations together with our finding, it may be general phenomenon that the stimulus which augments the proliferation rate of primordial cells is somehow transmitted even to those cells that are not in direct contact with the inductive tissue.

It is of interest to compare the results of our experiment with studies done on the human anencephalic fetuses whose adenohypophysial primordium often fails to contact with the brain [2–4, 8]. The adenohypophysis in anencephalic babies is extremely variable in size [2]. The blood sinusoids in the anencephalic hypophysis are much greater than in normal glands [8]. This means that a mere comparison of the organ weights in question is insufficient for evaluation of tissue growth. Therefore, the volume of the anencephalic adenohypophysis was estimated after correction for the amount of blood in the sinusoids and disclosed that the adenohypophysial malgrowth in anencephaly was not observable until 7 months of fetal age [4]. In view of the data indicating that the brain-adenohypophysial relationship develops rather normally at the early fetal stage [19], initial growth of the adenohypophysis may take place at a normal rate in anencephalic babies. Further studies are necessary to investigate to what extent the brain is involved in growth of the human adenohypophysis in congenital anomalies.

The developing adenohypophysial primordium of fetal rats can be roughly divided into two parts on the basis of the rate of cell proliferation [15]. The higher rate of cell proliferation in the dorsal (adneural) half of the primordium was already discussed as above in relation to the brain. The ventral half of the adenohypophysial primordium, on the other hand, has only a small number of proliferating cells. Does this mean that ventrally located mesenchyme inhibits proliferation of adenohypophysial primordial cells? This possibility is unlikely because 1) removal of mesenchyme failed to augment the proliferative rate of adenohypophysial cells, and 2) transplantation of neural tissue to the ventral part of the primordium did not change the incidence of cell proliferation. At present it is not known why those ventrally situated cells are insensitive in proliferation to the neural stimulus introduced by transplantation. Apart from the exact mechanism of such cell proliferation, it seems of interest that most types of hormone-producing cells first appear in the ventral half of the developing adenohypophysis in the rat [11, 22]. The nature of the BrDU-labelled cells is not known since no cells are reactive to antisera to adenohypophysial hormones at the developmental stage examined. In a prolonged culture experiment, we have observed that some of these labelled cells differentiated to LH, ACTH and PRL cells (Shirai and Watanabe, unpublished observation).

In the present transplantation experiments, our interest was focused on the diencephalic floor which is destined to make contact with the adenohypophysial rudiment. It is of interest to inquire whether only this region of the brain or other parts of the brain also have a stimulatory influence on proliferation of adenohypophysial primordial cells. The size of adenohypophysial explants co-cultured with part of the telencephalic roof was remarkably smaller than that of cultures kept with the anterior or posterior diencephalic floor [5]. Our most recent experiments have shown that brain tissues other than the diencephalic floor had little effect on cell proliferation of the adenohypophysial primordium [16].

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